

miR-200c as a Biomarker for 5-FU Chemosensitivity in Colorectal Cancer

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Abstract—5-FU is a chemotherapeutic agent that has been used in colorectal cancer (CRC) treatment. However, it is usually associated with the acquired resistance, which decreases the therapeutic effects of 5-FU. miR-200c is involved in chemotherapeutic drug resistance, but its mechanism is not fully understood. In this study, the effect of inhibition of miR-200c in sensitivity of HCT-116 CRC cells to 5-FU was evaluated. HCT-116 cells were transfected with LNA-anti-miR-200c for 48 h. mRNA expression of miR-200c was evaluated using quantitative real-time PCR. The protein expression of phosphatase and tensin homolog (PTEN) and E-cadherin were analyzed by western blotting. Annexin V and propidium iodide staining assay were applied for apoptosis detection. The caspase-3 activation was evaluated by an enzymatic assay. The results showed LNA-anti-miR-200c inhibited the expression of PTEN and E-cadherin protein, apoptosis and activation of caspase 3 compared with control cells. In conclusion, these results suggest that miR-200c as a prognostic marker can overcome to 5-FU chemoresistance in CRC.

Keywords—Colorectal cancer, miR-200c, 5-FU resistance, E-cadherin, PTEN.

I. INTRODUCTION

COLORECTAL cancer (CRC) as one of the most prevalent human malignancies in the modern world remains a serious cause of death among all cancer-related deaths [1]. Despite overall advances in treatment of diseases, CRC remains a main health issue leading to a mortality rate of approximately 5% in the developed countries [2]. 5-Fluorouracil (5-FU) is widely used to treat CRC and is still the main chemotherapy [3]. 5-FU, as a precursor of UTP and dTTP, interferes with the metabolism of DNA and RNA. Prolonged exposure of 5FU to cancer cells may cause chemoresistance. Resistance to 5-FU is a major clinical complication following the successful treatment of CRC [4]-[7]. It has not yet been clearly explained why cancer treatment becomes ineffective over time; however, it was shown that cancer cells become resistant via certain mechanisms such as alteration in drug target, drug inactivation, influx and efflux of drugs in the cells, processing of drug-induced damage, and evasion of apoptosis [8].

miRNAs are non-coding single strand RNAs with a length of 18–24 nucleotides. The first products of microRNAs, pri-

microRNAs, are cleaved by Drosha to release pre-microRNAs in the nuclear compartment. The complex is translocated into the cytoplasm by exportin-5. Pre-microRNAs are cleaved by Dicer into the mature microRNAs [9]. These small RNAs play important roles in various biological processes and downregulate the expression of gene at the post-transcriptional level. The complementary base-pairing between miRNA and their target mRNA, which causes degradation of mRNA or translation disturbance, depends on the degree of miRNA-mRNA complementarity [10]. The miR-200 family has five members consisting of miR-200a, miR-200b, miR-200c, miR-429, and miR-141. miR-200c was demonstrated to be downregulated in a variety of human cancers [11]-[13]. Cochrane et al. found that chemosensitivity to paclitaxel considerably increased following the transfection of miR-200c into endometrial cancer cells [14]. Similarly, Ceppi et al. demonstrated that the upregulation of miR-200c maintained the sensitivity of non-small cell lung cancer (NSCLC) cells to cisplatin and cetuximab [15]. miR-200c decreases during tumor progression and serves as a key inhibitor for tumor cell invasion, metastasis, and epithelial-to-mesenchymal transition (EMT) [16]. miR-200c is associated with cancer progression and chemotherapeutic drug resistance, but the exact mechanism remains unclear [17].

Our aim was to evaluate the effect of LNA-anti-miR-200c transfection on the expression of E-cadherin and PTEN, as downstream targets of miRNA-200c, as well as the sensitivity of HCT-116 cells to 5-FU.

II. METHODS

A. Cell Culture

HCT-116 cell line (human CRC) was purchased from the Pasteur Institute (Tehran, Iran). The cells were cultured in DMEM medium (Gibco, Invitrogen, USA) containing 15% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Invitrogen). HCT-116 cells were incubated in a humidified environment at 37 °C and 5% CO₂.

B. Cell Transfection

The sequence of miR-200c was TCCATCATTACCCGGCA-GTA (accession number MIMAT0000617). miRCURY LNA microRNA InhibitorTM for hsa-miR-200c and the scrambled LNA were purchased from the Exiqon (Copenhagen, Denmark). The 5' ends of both oligonucleotides were labeled with fluorescent dye, 6-FAM. Transfection of HCT-116 cells was conducted according to the manufacturer's instructions by the X-treme GENE siRNA Transfection ReagentTM (Roche, Mannheim, Germany).

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Briefly, 5×10^5 cells were cultured in six-well culture plates (Spl, Korea) containing 1.8 ml DMEM per well without antibiotic and FBS. Five μ L of X-treme GENE siRNA Transfection ReagentTM was mixed with 50 pmol miRCURY LNA microRNA InhibitorTM in 200 μ L Opti-MEMI MediumTM (Gibco, Paisley, UK) and then incubated at room temperature for 15 min. The mixture was then added to the cells and shaken carefully to ensure even spreading over the entire plate surface. After an 8-h incubation, the antibiotics and FBS were added and the cells were incubated for the 24, 48, and 72 h [18]. This method was applied for cells transfected with scrambled-LNA. Assessment of the transfection was conducted by fluorescent microscopy and Flow cytometry.

C. Cell Viability Assay

The inhibitory effects of 5-FU on cell growth were investigated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). Five thousand HCT-116 cells were seeded in 96-well tissue culture plates, and various concentrations of 5-FU (0-20 μ M) added. After 24 and 48-h incubation, 10 μ L of MTT solution was added to each well and incubated for 4 h at 37 °C. Cell culture medium was removed and then 100 μ L of DMSO (Sigma-Aldrich) added. The optical density (OD) was measured by an ELISA reader at 570-nm wavelength. To evaluate the effect of miR-200c on 5-FU sensitivity of HCT-116, the cells were transfected with LNA-anti-miR-200c, 5-FU was added to the wells, and cell viability was analyzed by MTT assay.

D. Real Time PCR

Total RNA was extracted from HCT-116 cells using the RNX- Plus solution (Sinaclone, Iran). RNA concentration and purity were measured by nanodrop. 2 μ g of total RNA was used for complementary DNA (cDNA) synthesis from miRNA according to the manufacturer (Parsgenom, Tehran, Iran) instructions. Real time-PCR was conducted with SYBR green master mix and specific miR-200c primers. Thermal cycle for mixture consisted of an initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 5 s, annealing at 62 °C for 20 s, and an extension for 30 s at 72 °C. The levels of mRNA expression were normalized to 5S rRNA. Relative expression was reported by $2^{-\Delta\Delta CT}$ method.

E. Western Blot Analysis

PTEN and E-cadherin protein expression was investigated by Western blotting. HCT-116 cells were lysed in 0.2 ml of RIPA buffer (Santa Cruz, USA) and centrifuged at 13,000 g for 20 min at 4 °C. The protein concentration was measured by Bradford assay. For immunoblotting, 50 μ g of each sample was loaded into wells and separated by SDS-PAGE. Then, the proteins were transferred onto a nitrocellulose membrane. The membrane was incubated with primary antibodies: Rabbit monoclonal anti- PTEN (ab32199, 1:7000) and Rabbit monoclonal anti- E-cadherin (ab40772, 1:7000). Then, the membranes were incubated at room temperature for 1 h with the secondary antibody (anti-rabbit antibody conjugated with horse-radish peroxidase). β -Actin was used as a loading

control. Immunoreactivity was visualized using an ECL kit (Amersham, USA) and quantified by densitometric analysis using ImageJ software (National Institute of Health, Bethesda, MD, USA) [19].

F. Apoptosis Assay

Annexin V-FITC/PI assay was used to detect apoptosis induced by LNA-anti-miR-200c in HCT-116 cancer cells. HCT-116 cells were seeded in 6-well plates at a 200000 cells/well density. After treatment procedure, the adherent cells were trypsinized and centrifuged at 1500 g for 5 min. Afterward, the cells were stained with annexin V and PI according to the manufacturer's instructions. Annexin V-FITC detects the Phosphatidylserine during apoptosis, and PI distinguishes necrotic cells. The cells were analyzed using a FACS caliber cytometer (Becton Dickinson, San Diego, CA, USA) and CellQuest software.

G. Caspase 3 Activity Assay

The activation of caspases 3 was evaluated using caspase 3 colorimetric assay kit (Cambridge, MA, USA). Briefly, the treated HCT-116 cells were trypsinized and centrifuged at 1800 g for 10 min. The cell pellet was washed in ice-cold PBS and lysed in 50 μ L of lysis buffer and centrifuged at 10000 g for 1 min at 4 °C. After the protein concentration was determined by a Bradford assay, 100 μ g of the proteins from each sample was added to 50 μ L of reaction buffer containing DTT and caspase-3 substrate (DEVD-pNA), and incubated at 37 °C for 1-2 h. The activity of caspase-3 in cell lysate was measured at 405-nm wavelength in the plate reader.

H. Statistical Analysis

Data were expressed as mean $\pm$ standard error of the mean (SEM). One-way ANOVA and Tukey's posthoc tests were used to determine the statistical significance of inter-group differences. $P < 0.05$ was considered statistically significant.

III. RESULTS

A. Effect of miR-200c on 5-FU Sensitivity of HCT-116 Cells

We investigated the effect of various concentrations of 5-FU (1-20 μ M) on HCT-116 cells viability by MTT assay. As illustrated in Fig. 1, 5-FU decreased the percentage of viable cells compared with the untreated cell in a dose and time-dependent manner. 5-FU displayed significant cytotoxicity at 5- μ M concentration for 48 h ($p < 0.001$). The 50% inhibition concentration (IC_{50}) values were derived 10 μ M after 48 h. In addition, the sensitivity of HCT-116 cells transfected with LNA-anti-miR-200c to 5-FU decreased compared with that of the untransfected cells. The IC_{50} of HCT-116 cells transfected with anti-miR-200c was 1.50 times higher ($p < 0.01$) than that of untransfected HCT-116 cells.

B. Effect of LNA-Anti-miR on miR200c Expression

For inhibition of miR-200c, HCT-116 cells were transfected with the miRCURY LNA miR inhibitor. The transfection efficiency was determined using flow cytometry and fluorescence microscopy. We obtained transfection efficiency

of HCT-116 cells with LNA-anti miR-200c to be about 70% (data not shown). The expression of miR-200c was evaluated at 12, 24, 48, and 72 h after transfection by reverse transcriptase microRNA real time-PCR in HCT-116 cells transfected with LNA-anti-miR, scrambled LNA, and untreated HCT-116 cells. In all four time intervals, the expression of miR-200c was considerably lower in the LNA-anti-miR group than the untreated groups. However, there was no significant difference between scrambled LNA-transfected group and the untreated cells (Fig. 2).

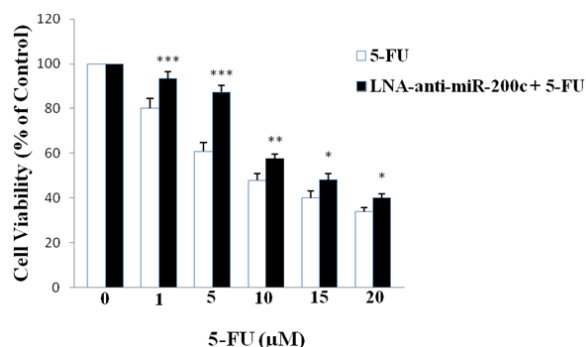


Fig. 1 Effect of LNA-anti-miR-200c on 5-FU sensitivity of HCT-116 cells. Data are represented as mean $\pm$ SEM (n = 8). * P < 0.05, ** P < 0.01 and *** P < 0.001 versus cells treated with 5-FU

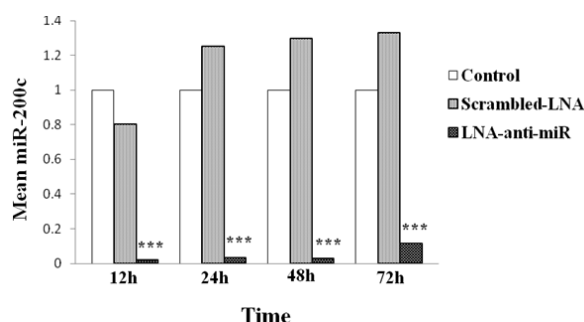


Fig. 2 Effect of LNA-anti-miR on miR200c expression. All data were normalized to levels of 5S rRNA. Data are represented as mean $\pm$ SEM. *** P < 0.001 versus control group

C. Effect of miR-200c Inhibition on PTEN and E-Cadherin Protein Expression

Then, we examined the expression of PTEN and E-cadherin, two downstream targets of miR-200c, by western blot. Immunoblotting analysis revealed that the levels of PTEN and E-cadherin were reduced by knockdown of miR-200c in HCT-116 cells. Fig. 3 shows that depletion of miR-200c decreases 5-FU-stimulated PTEN and E-cadherin expression.

D. Effect of miR-200c on Apoptosis

To investigate the effect of miR-200c inhibition on apoptosis, the HCT-116 cells were stained with Annexin-V. We examined 5-FU-induced apoptosis in HCT-116 cells following transfection with LNA-anti-miR-200c. We found that miR-200c inhibition decreased the apoptosis following

treatment with 5-FU, but apoptosis in 5-FU-treated cells was higher than that in the control group (Fig. 4).

E. Effect of miR-200c Inhibition on Caspase 3 Activity

The effect of miR-200c on 5-FU-mediated apoptosis was confirmed by caspase 3 activity assay. miR-200c inhibition reduced the caspase 3 activity following treatment with 5-FU compared with 5-FU-treated HCT-116 cells (Fig. 5).

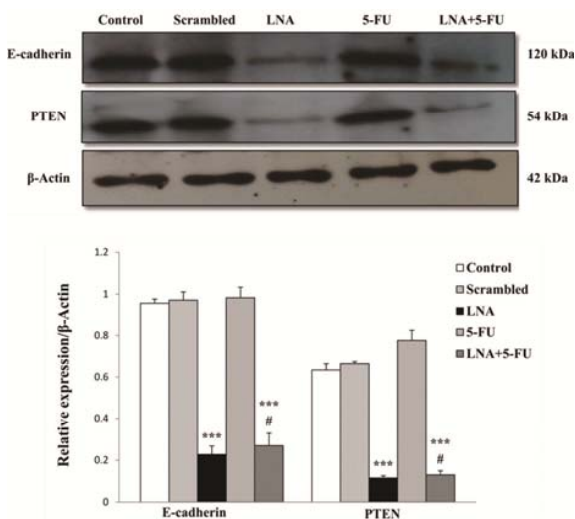


Fig. 3 Effect of miR-200c inhibition on PTEN and E-cadherin protein expression. *** P < 0.001 versus control cells and # P < 0.001 versus 5-FU. (n = 3)

IV. DISCUSSION

CRC-associated morbidity and mortality are high worldwide. Metastasis is the main cause of death in CRC patients [20]. Therefore, for successful treatment, surgery or radiotherapy can be done alongside chemotherapy. 5-FU is considered the main chemotherapy drug of choice to treat CRC and reduce the annual probability of recurrence and mortality. However, resistance to chemotherapy drugs is a major obstacle to efficient treatment of cancer [21]. The role of miRNAs in chemoresistance was demonstrated [22]. Emerging evidence suggests that the loss of miR-200c expression is involved in the cancer cell resistance to chemotherapy drugs [23].

The role of miR-200c in chemoresistance in esophageal and breast cancer was demonstrated, but its role in chemoresistance in CRC has not yet been explained [16], [23]. In this study for the first time, we showed that the inhibition of miR-200c correlated with the acquired resistance of CRC cells (HCT-116) to 5-FU. To achieve this purpose, we used LNA-anti miR-200c to knockdown miR-200c in order to evaluate the HCT-116 colon cancer cells response to 5-FU. In the current study, we investigated the effect of miR-200c inhibition on apoptosis and the expression of PTEN and E-cadherin protein. Our results showed that LNA-anti-miR-200c suppressed the expression of miR-200c, PTEN, and E-cadherin compared with the control cells. Furthermore, we

observed that LNA-anti-miR-200c could promote cell proliferation through inhibition of caspase 3 activity and reduction of apoptosis that is mediated by PTEN expression.

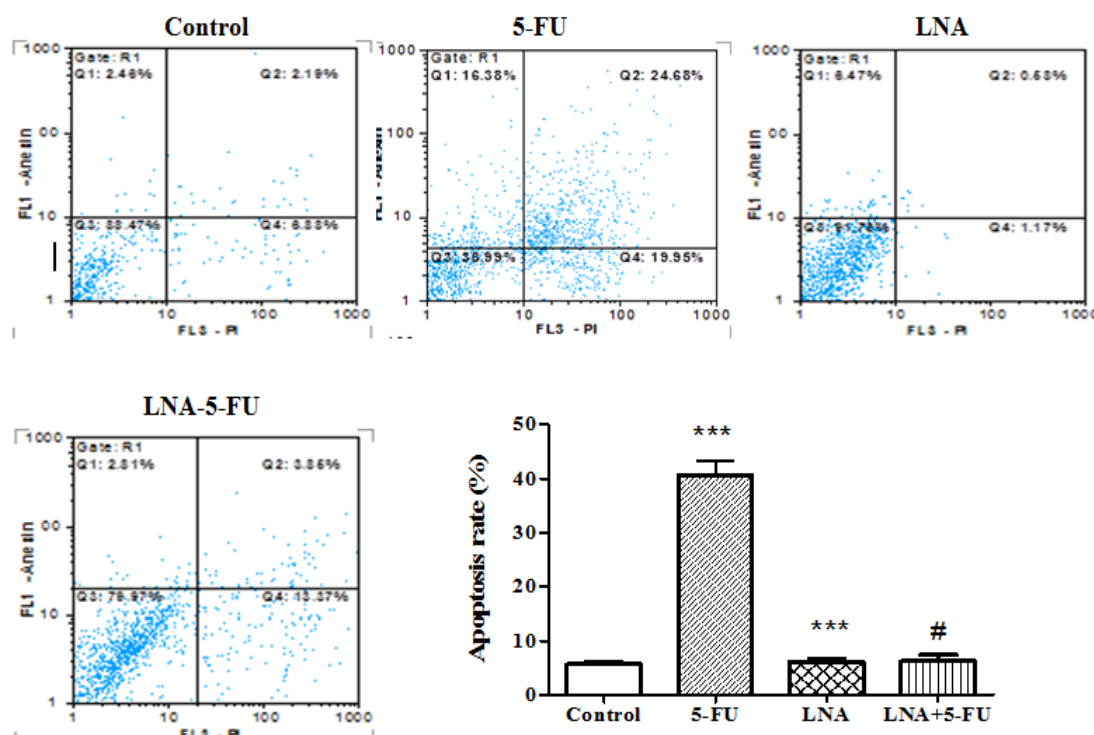


Fig. 4 Effect of miR-200c inhibition on HCT-116 cells apoptosis. Data are presented as the mean $\pm$ SEM. *** $p < 0.001$ versus control and # $P < 0.001$ versus 5-FU

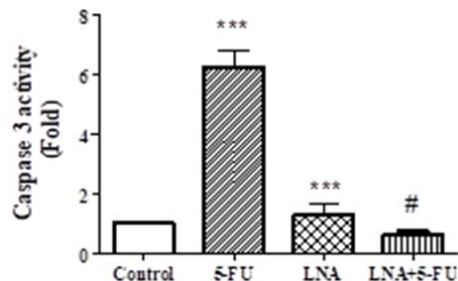


Fig. 5 Effect of miR-200c inhibition on caspase 3 activity. Caspase 3 activity was decreased by knockdown of miR-200c in HCT-116 cells. Data are represented as mean $\pm$ SEM (n = 3). *** $P < 0.001$ versus control cells and # $P < 0.001$ versus 5-FU

PTEN is a tumor suppressor gene located in the cytoplasm and is responsible for lipid and protein dephosphorylation [24]. PTEN is an essential factor in the suppression of tumor cell proliferation, cell migration, and apoptosis [10]. Downregulation of the PTEN gene is prevalent in various tumors, such as those in brain, endometrium, colorectal, skin, prostate, and breast cancers [25]. A study has shown that miR-200c decreases the expression of PTEN in pituitary adenoma [26]. In contrast, a study by Chen et al. revealed that miR-200c inhibited doxorubicin resistance in MCF-7 breast cancer cells by suppressing Akt signaling via upregulation of PTEN and E-cadherin [23]. It seems that miR-200c can upregulate

the expression of PTEN indirectly through enhancing E-cadherin expression. Furthermore, it should be noted that PTEN inactivates the AKT signaling pathway by phosphatidylinositol triphosphate dephosphorylation and subsequent inhibition of AKT phosphorylation [27]-[29].

E-cadherin as a transmembrane protein plays a critical role in cell adhesion and is involved in transmitting intracellular chemical signals. It is one of the major downstream regulators of miRNA-200c contributing to EMT, which is also important to inhibit tumor invasion and proliferation as well as to induce cell apoptosis [9], [30], [31]. Li et al. demonstrated that restoring E-cadherin-mediated cell-cell adhesion enhanced PTEN protein levels by increasing PTEN protein stability and suppressing degradation in human breast cancer cells [10].

Consistent with our findings, a study has shown that ectopic expression of miR-200c inhibits EMT, invasion, and metastasis through blocking ZEB1 expression and increasing the E-cadherin/TA-p73/p63 expression in lung carcinoma cell lines [32].

Evidence suggests that ZEB1 plays an important role in the EMT of CRC cells. miR-200c is able to prevent EMT process through suppression of several targets, such as ZEB1/2. miR-200 suppresses the expression of ZEB by binding to its 3' UTRs and inhibits cell invasion [33], [34].

It has also been shown that PTEN level decreases in cetuximab-resistant cell lines (HCC827-CR), leading to the

activation of PI3K/Akt signaling, while upregulation of PTEN significantly caused reduction in Akt activity and restored drug sensitivity. PTEN downregulation seems to provoke the acquired resistance of NSCLC to cetuximab by activation of PI3K/Akt signaling [35]. Moreover, upregulation of miRNA-200c restored the sensitivity of non-small cell lung cancer (NSCLC) cells to cisplatin and cetuximab [15]. miRNA-200c is important in the EMT and metastasis in different types of cancers. Several studies showed that downregulation of miRNA-200c occurs in a variety of human cancer types [12], [13], [23], [36]. miR-200c inhibits EMT, proliferation, metastasis, resistance to chemotherapy drugs and also contributes significantly to the development of drug resistance in cancer cells [37]-[39].

Recently, it was demonstrated that the cells that lack miR-200c expression are more prone to invasion into the lymphatic system and blood. Naïve CCL227 cell-derived exosomes contain high levels of miR-200c, while the 5-FU-resistant CCL227 exosomes are free of miR-200c and accelerated circular chemorepellent-induced defects (CCIDs) formation in the blood endothelial cell (BEC) compared to naïve CCL227. miR-200c inhibits CCIDs formation through downregulation of SNAI, ZEB2, and TWIST expression in the BECs [40].

Another study has shown that the serum levels of miR-155, miR-200c, and miR-201 can predict relapse and distant metastasis, chemoresistance, and poor prognosis in colon cancers [41]. It was demonstrated that curcumin chemosensitizes CRC cells to 5-FU via the upregulation of miR-200c in 5-FU cell lines [42]. Soubani et al. reported that the loss of miR-200 family and PTEN expression led to aggressive behavior of pancreatic cancer cells [43].

Overall, the inhibition of miR-200c correlated with the acquired resistance of CRC cells (HCT-116) to 5-FU with decreasing the levels of E-cadherin and PTEN protein. This study suggests that miR-200c can be a possible therapeutic agent, particularly in combination with anti-cancer chemotherapy agents.

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